

# Primary structure of three cationic peptides from porcine neutrophils

## Sequence determination by the combined usage of electrospray ionization mass spectrometry and Edman degradation

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The primary structure of three major cationic peptides from porcine neutrophils has been determined. The sequencing was made by the combined use of electrospray ionization mass spectrometry and Edman degradation. The determined sequences unambiguously show that these peptides can not be considered as defensins.

Peptide from neutrophil; Primary structure; Electrospray ionization mass spectrometry

## 1. INTRODUCTION

The study of structural features of cationic peptides, including ones from mammalian blood neutrophils, is of particular interest due to their known antimicrobial, antifungal and antiviral activities. These properties, together with their low toxicity and absence of immunoreactivity, have led us to consider the cationic peptides from neutrophils as novel types of antibiotics [1].

In the present study, three major peptides from porcine blood neutrophils were purified and their amino acid sequences determined. The sequencing was carried out by the combined use of electrospray ionization mass spectrometry (ESI MS) and Edman degradation. The principal advantages of ESI MS are the determination of molecular masses of peptides or proteins with high accuracy, and the direct qualitative analysis of peptide mixtures without their preliminary fractionation [2,3]. The approach proposed might be especially useful for sequence determination in homologous proteins and peptides.

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**Abbreviations:** PNP, porcine neutrophil peptide; ESI MS, electrospray ionization mass spectrometry; HFBA, heptafluorobutyric acid; Cys\*, pyridylethylcysteine residue.

## 2. MATERIALS AND METHODS

A freshly obtained sample of porcine blood leukocytes was homogenized in 10% acetic acid followed by centrifugation at  $25,000 \times g$  for 1 h. The supernatant was subjected to 24 h dialysis against 5 vols. of 10% acetic acid in Sigma 250-7U tubing. The lyophilized dialyzate was separated on a  $5 \times 100$  cm Acrylex P 10 column equilibrated in 5% acetic acid. The low molecular weight fraction was collected and lyophilized, yielding the total porcine neutrophil peptide (PNP) preparation.

The total PNP preparation was separated by reverse-phase (RP) HPLC on a Milikhrom liquid chromatograph (Nauchpribor, Russian Federation) on  $2 \times 62$  mm columns packed with Nucleosil 5 C<sub>18</sub> (Macherey-Nagel) by a 20–35% gradient of acetonitrile in acetonitrile/0.1% TFA mixture. The eluate was detected at 210 nm.

Mass spectra were recorded on a time-of-flight (TOF) mass reflectron coupled with an electrospray ion source [4]. Samples were redissolved in 2% acetic acid/acetonitrile 1:1 (v/v), and injected at a flow rate of 2  $\mu$ l/min. The time of spectra registration was 0.5 min. Less than 100 pmol of the sample was required for spectra recording.

Individual peptides were reduced by  $\beta$ -mercaptoethanol and alkylated by 4-vinylpyridine in accordance with [5] with some modifications. The reaction mixtures were desalted by RP-HPLC. The number of cysteine residues was calculated from the difference between the masses of intact and pyridylethylated peptides.

Amino acid composition was determined using a model D-500 amino acid analyzer (Durrum). Amino acid sequences were determined using a model 816 automatic peptide sequencer (Knauer) equipped with a model 120A PTH analyzer (Applied Biosystems).

The reduced and alkylated peptides were digested by pepsin in 1% acetic acid at 37°C; the enzyme–substrate ratio was 1 : 50. Digestion products were separated by RP-HPLC.

Peptide hydrolysis was carried out by heptafluorobutyric acid (HFBA) vapors, as described in [6] with some modifications.

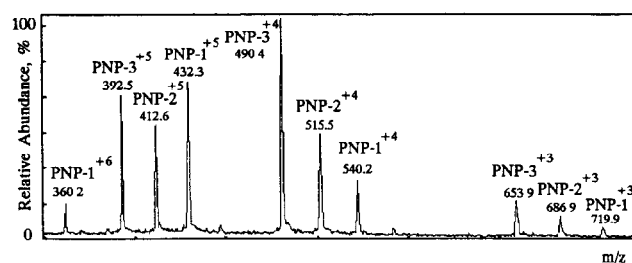


Fig. 1. Mass spectrum of the total preparation of peptides from porcine neutrophils. The charges of corresponding quasimolecular ions are shown above the peak designations.

### 3. RESULTS AND DISCUSSION

The ESI mass spectrum (Fig. 1) and the chromatogram (Fig. 2) of the total PNP preparation show that it contains three major peptides (PNPs 1–3) (peptides are numbered in accordance with their elution order). Molecular masses and amino acid composition data for individual peptides are shown in Table I.

Treatment of PNPs by the Ellman's reagent showed the absence of free SH-groups. After peptide reduction followed by pyridylethylation of the formed cysteine residues, the molecular weight of each of the PNPs was increased by 424.6 Da, which shows that each of the peptides contains four cysteine residues involved in two inter-molecular disulphide bonds.

Mass spectrometric analysis of the products formed

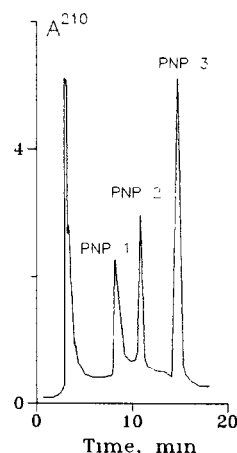


Fig. 2. Chromatogram of the total preparation of the peptides from porcine neutrophils. Experimental conditions are given in Section 2.

after acetylation of PNPs by the acetic anhydride/methanol mixture showed the formation of mono- and double acetyl derivatives without any methyl ester ones. As was previously found in model experiments, all free -COOH groups in peptides undergo complete esterification under these conditions (data not shown). Consequently all the PNPs have blocked C-terminals.

For mass spectrometrical mapping and sequencing, PNPs were subjected to reduction of disulphide bridges with subsequent pyridylethylation. The treated PNPs

Table I  
Main characteristics for the peptides from porcine neutrophils

Peptide	$M_r$ (Da)		Amino acid composition measured (obtained from sequence data)	Mass spectrometric mapping data	
	Measured	Calculated		$M_r$ (Da)	Variants of amino acid composition*
PNP-1	$2156.6 \pm 0.5$	2155.7	Gly 3.1 (3)	P <sub>1</sub> 557.7	G <sub>2</sub> R <sub>2</sub> L, (VG <sub>3</sub> RL)
			Val 2.1 (2)	P <sub>2</sub> 389.3	CY
			Leu 1.1 (1)	P <sub>3</sub> 841.9	CR <sub>3</sub> F, (ZGVR <sub>2</sub> F, G <sub>2</sub> V <sub>2</sub> RCF)
			Tyr 0.8 (1)		
			Phe 0.9 (1)		
			Arg 5.8 (6)		
PNP-2	$2057.6 \pm 0.5$	2056.5	Cys <sub>2</sub> 2 (2)	P <sub>4</sub> 844.8	V <sub>2</sub> Z <sub>2</sub> GR, (VR <sub>2</sub> Z <sub>2</sub> )
			Gly 4.1 (4)	P <sub>1</sub> 458.5	G <sub>3</sub> RL
			Val 2.1 (2)		
			Leu 1.1 (1)	P <sub>2</sub> 389.3	CY
			Tyr 0.8 (1)		
			Phe 1.0 (1)	P <sub>3</sub> 841.9	CR <sub>3</sub> F, (ZGVR <sub>2</sub> F, G <sub>2</sub> V <sub>2</sub> RC)
PNP-3	$1957.5 \pm 0.5$	1956.5	Arg 4.9 (5)		
			Cys <sub>2</sub> 2 (2)	P <sub>4</sub> 844.8	V <sub>2</sub> Z <sub>2</sub> GR, (VR <sub>2</sub> Z <sub>2</sub> )
			Gly 2.1 (2)	P <sub>1</sub> 557.7	G <sub>2</sub> R <sub>2</sub> L, (VG <sub>3</sub> RL)
			Val 1.0 (1)		
			Leu 1.6 (1)	P <sub>2</sub> 389.3	CY
			Tyr 0.8 (1)		
			Phe 1.0 (1)	P <sub>3</sub> 841.9	CR <sub>3</sub> F, (ZGVR <sub>2</sub> F, G <sub>2</sub> R <sub>2</sub> RC)
			Arg 4.9 (5)		
			Ile 0.9 (1)	P <sub>4</sub> 645.6	C <sub>2</sub> VI
			Cys <sub>2</sub> 2 (2)		

\*C = pyridylethylcysteine residues; alternative variants of amino acid composition are shown in parentheses.



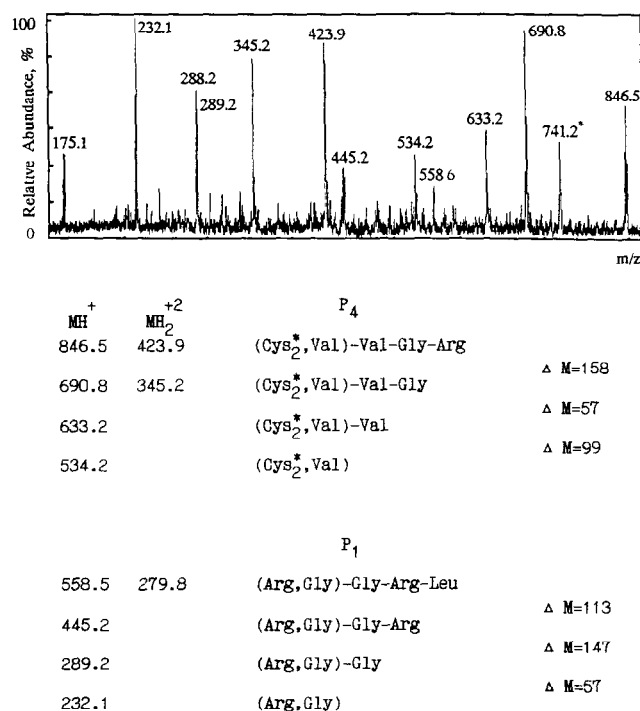


Fig. 5. Mass spectrum of the hydrolyzate of a mixture of P<sub>1</sub> and P<sub>4</sub> fragments formed after HFBA vapor treatment. The fragment ion of the P<sub>4</sub> quasimolecular ion formed by Cys<sup>\*</sup> loss ( $M = 105.2$ ) is marked by an asterisk. The amino acid composition of the N-terminal parts of P<sub>1</sub> and P<sub>4</sub>, calculated from their molecular masses, are shown in parentheses.

MS technique. Mass spectrometric analysis of partial enzyme digests of a polypeptide chain enables one to

discover the structural fragments and to determine their localization. Comparison of masses of structural fragments of homologous polypeptides allows the localization of the fragments with amino acid substitutions. Mass spectrometric analysis of peptide hydrolyzates resulting from HFBA vapor treatment enables the partial C-terminal sequences to be determined. ESI MS is a good supplement to the Edman sequencing technique, the use of which often leaves C-terminal sequences undetermined. At the same time, the ESI MS technique does not allow the determination of Leu and Ile positions in the polypeptide sequence, which must therefore be determined by other methods.

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